

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 01/08/03	2. REPORT TYPE Final Report	3. DATES COVERED 03/01/97 – 02/28/98		
4. TITLE AND SUBTITLE Optimization of Biosensors by Directed Evolution		5a. CONTRACT NUMBER N00014-97-1-0431		
		5b. GRANT NUMBER		
		5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Fierke, Carol A.		5d. PROJECT NUMBER		
		5e. TASK NUMBER		
		5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke Univ. Med. Ctr (previous) Biochemistry Department Durham, NC 27710		8. PERFORMING ORGANIZATION REPORT NUMBER		
University of Michigan (current) Chemistry Department 930 N. University Ann Arbor, MI 48109				
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research 800 N. Quincy St. Arlington, VA 22217-5000		10. SPONSOR/MONITOR'S ACRONYM(S) ONR		
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
<p style="text-align: right;">20030123 046</p> 14. ABSTRACT <p>The objective of this work is to develop methodologies for the optimization of field-deployable optical biosensors. We used these Defense University Research Instrumentation Program funds to purchase a Perkin-Elmer GeneAmp PCR System 2400 thermal cycler and a SpectraMax Plus plate reader from Molecular Dynamics. We used the plate reader to develop faster assays for characterizing carbonic anhydrase (CA) variants. We used the thermal cycler to prepare a large library of CA variants. We then completed multiple rounds of selection for variants with enhanced zinc specificity using phage display. We successfully prepared variants with altered metal specificities using these methods. These variants can be used to optimize a carbonic anhydrase-based metal ion biosensor.</p>				
15. SUBJECT TERMS Thermal cycler, plate reader, DNA libraries, binding, phage display				
16. SECURITY CLASSIFICATION OF: a. REPORT Unclass.		17. LIMITATION OF ABSTRACT Unclass.	18. NUMBER OF PAGES 43	19a. NAME OF RESPONSIBLE PERSON Carol A. Fierke
				19b. TELEPHONE NUMBER (Include area code) 734-936-2678

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

1. REPORT DATE. Full publication date, including day, month, if available. Must cite at least the year and be year 2000 compliant, e.g. 30-06-1998; xx-06-1998; xx-xx-1998.

2. REPORT TYPE. State the type of report, such as final, technical, interim, memorandum, master's thesis, progress, quarterly, research, special, group study, etc.

3. DATES COVERED. Indicate the time during which the work was performed and the report was written, e.g., Jun 1997 - Jun 1998; 1-10 Jun 1996; May - Nov 1998; Nov 1998.

4. TITLE. Enter title and subtitle with volume number and part number, if applicable. On classified documents, enter the title classification in parentheses.

5a. CONTRACT NUMBER. Enter all contract numbers as they appear in the report, e.g. F33615-86-C-5169.

5b. GRANT NUMBER. Enter all grant numbers as they appear in the report, e.g. AFOSR-82-1234.

5c. PROGRAM ELEMENT NUMBER. Enter all program element numbers as they appear in the report, e.g. 61101A.

5d. PROJECT NUMBER. Enter all project numbers as they appear in the report, e.g. 1F665702D1257; ILIR.

5e. TASK NUMBER. Enter all task numbers as they appear in the report, e.g. 05; RF0330201; T4112.

5f. WORK UNIT NUMBER. Enter all work unit numbers as they appear in the report, e.g. 001; AFAPL30480105.

6. AUTHOR(S). Enter name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. The form of entry is the last name, first name, middle initial, and additional qualifiers separated by commas, e.g. Smith, Richard, J, Jr.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES). Self-explanatory.

8. PERFORMING ORGANIZATION REPORT NUMBER. Enter all unique alphanumeric report numbers assigned by the performing organization, e.g. BRL-1234; AFWL-TR-85-4017-Vol-21-PT-2.

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES). Enter the name and address of the organization(s) financially responsible for and monitoring the work.

10. SPONSOR/MONITOR ACRONYM(S). Enter, if available, e.g. BRL, ARDEC, NADC.

11. SPONSOR/MONITOR'S REPORT NUMBER(S). Enter report number as assigned by the sponsoring/monitoring agency, if available, e.g. BRL-TR-829; -215.

12. DISTRIBUTION/AVAILABILITY STATEMENT. Use agency-mandated availability statements to indicate the public availability or distribution limitations of the report. If additional limitations/restrictions or special markings are indicated, follow agency authorization procedures, e.g. RD/FRD, PROPIN, ITAR, etc. Include copyright information.

13. SUPPLEMENTARY NOTES. Enter information not included elsewhere such as: prepared in cooperation with; translation of; report supersedes; old edition number, etc.

14. ABSTRACT. A brief (approximately 200 words) factual summary of the most significant information.

15. SUBJECT TERMS. Key words or phrases identifying major concepts in the report.

16. SECURITY CLASSIFICATION. Enter security classification in accordance with security classification regulations, be.go. U, C, S, etc. If this form contains classified information, stamp classification level on the top and bottom of this page.

17. LIMITATION OF ABSTRACT. This block must be completed to assign a distribution limitation to the abstract. Enter UP (Unclassified Unlimited) or SAR (Same as Report). An entry in this block is necessary if the abstract is to be limited.

FINAL REPORT

Grant#: N00014-97-1-0431

PRINCIPAL INVESTIGATOR: Dr. Carol Ann Fierke

INSTITUTION: University of Michigan (current)
Duke University Medical Center (previous)

E-MAIL: fierke@umich.edu

GRANT TITLE: Optimization of Biosensors by Directed Evolution

AWARD PERIOD: 01 March 1997 - 28 February 1998

OBJECTIVE: This Defense University Research Instrumentation Program provides equipment funds for research and education. The objective of this work is to develop methodologies for the optimization of field-deployable optical biosensors. In particular, a carbonic anhydrase-based fiber optic metal ion biosensor will be enhanced by the preparation and use of enzyme variants.

APPROACH: We propose to buy a thermal cycler to facilitate the use of the polymerase chain reaction to amplify DNA for preparing CA variants. Furthermore, we also propose to buy a microplate reader to speed up characterization of CA variants, including the zinc affinity, metal ion specificity, and stability.

ACCOMPLISHMENTS: *Thermal cycler:* We purchased a Perkin-Elmer GeneAmp PCR System 2400 thermal cycler to facilitate the preparation of libraries of CA variants and subcloning of these variants. We prepared a large library ($\approx 10^9$) of CA variants with substitutions at amino acids near the zinc binding site. This library includes substitutions in the direct metal ligands (H94, H96 and H119), the "indirect" metal ligands (Q92, E117 and T199) and the hydrophobic pocket beneath the zinc binding site (F93, F95, W97, L118 and L120). We have previously shown that each of these elements affect the metal binding site, including metal affinity, metal equilibration kinetics and metal specificity. We developed methods to use sulfonamide affinity chromatography in the presence of various Zn/metal ratios to screen this phage library for variants with altered metal ion specificity. We completed multiple rounds of selection for variants with enhanced zinc specificity.

Plate reader: We have purchased the SpectraMax Plus plate reader from Molecular Dynamics with path check capabilities, a monochromator and the ability to measure absorbance in a cuvette. We have used this instrument to assay esterase activity of CAII and the catalytic activity of other enzymes.

We are continuing to work on the development of assays to measure metal ion specificity. Using our conventional methodology for assaying variants, we have determined metal ion affinities and catalytic activity for variants in both the histidine ligands and the hydrophobic pocket beneath the zinc binding site. These data indicate that it is possible to alter the metal ion specificity of carbonic anhydrase.

CONCLUSIONS: We demonstrated that the phage display methodology could successfully be used to identify carbonic anhydrase variants with altered metal specificities.

SIGNIFICANCE: The development of technology to rapidly prepare and screen CA variants for useful properties will significantly enhance the optimization a CA-based metal ion biosensor by increasing the number of variants that can be examined. These methodologies should also be useful for the screening and characterization of any large library.

PATENT INFORMATION: NONE

AWARD INFORMATION: NONE

PUBLICATIONS AND ABSTRACTS (for total period of the grant):

1. Lesburg, C. A., C.-c. Huang, D. W. Christianson, and C. A. Fierke. 1997. Histidine→Carboxamide Ligand Substitutions in the Zinc Binding Site of Carbonic Anhydrase II Alter Metal Coordination Geometry but Retain Catalytic Activity. *Biochemistry* 36:15780-15791.
2. Hunt, J. A. and C. A. Fierke. 1997. Selection of Carbonic Anhydrase Variants Displayed on Phage: Aromatic Residues in Zinc Binding Site Enhance Metal Affinity and Equilibration Kinetics. *J. Biol. Chem.* 272:20364-20372.
3. Thompson, R. B., B. P. Maliwal, and C. A. Fierke 1998. Expanded Dynamic Range of Free Zinc Ion Determination by Fluorescence Anisotropy. *Anal. Chem.* 70: 1749-1754.
4. Thompson, R. B., B. P. Maliwal, V. L. Feliccia, C. A. Fierke, and K. M. McCall. 1998. Determination of picomolar concentrations of metal ions using fluorescence anisotropy: biosensing with a "reagentless" enzyme transducer. *Anal. Chem.* 70: 4717-4723.
5. Hunt, J. A., M. Ahmed and C. A. Fierke. 1999. Metal Binding Specificity in Carbonic Anhydrase is Influenced by Conserved Hydrophobic Core Residues. *Biochemistry* 38: 9054-9060.

4. Hunt, J. A. and C. A. Fierke. 1997. Selection of Carbonic Anhydrase Variants Displayed on Phage: Aromatic Residues in Zinc Binding Site Enhance Metal Affinity and Equilibration Kinetics. *J. Biol. Chem.* 272:20364-20372.
5. Thompson, R. B., B. P. Maliwal, and C. A. Fierke 1998. Expanded Dynamic Range of Free Zinc Ion Determination by Fluorescence Anisotropy. *Anal. Chem.* 70: 1749-1754.
6. Thompson, R. B., B. P. Maliwal, V. L. Feliccia, C. A. Fierke, and K. M. McCall. 1998. Determination of picomolar concentrations of metal ions using fluorescence anisotropy: biosensing with a "reagentless" enzyme transducer. *Anal. Chem.* 70: 4717-4723.
7. Thompson, R. B., B. P. Maliwal, and C. A. Fierke. 1999. Selectivity and sensitivity of fluorescence lifetime-based metal ion biosensing using a carbonic anhydrase transducer. *Anal. Biochem* 267: 185-195.
8. Hunt, J. A., M. Ahmed and C. A. Fierke. 1999. Metal Binding Specificity in Carbonic Anhydrase is Influenced by Conserved Hydrophobic Core Residues. *Biochemistry* 38: 9054-9060.
9. Hunt, J. A., C. A. Lesburg, D. W. Christianson, R. B. Thompson, and C. A. Fierke. 2000. Active Site Engineering of Carbonic Anhydrase and its Application to Biosensors, *The Carbonic Anhydrases: New Horizons* (Chegwidden, W. R., Carter, N. D. and Edwards, Y. H., ed.). Birkhauser Verlag, Basel/Switzerland. 221-240.
10. Cox, J. D., J. A. Hunt, K. M. Compher, C. A. Fierke and D. W. Christianson. 2000. Structural Influence of Hydrophobic Core Residues on Metal Binding and Specificity in Carbonic Anhydrase II. *Biochemistry* 39: 13687-13694.